CONTINUOUS ZONE ELECTROPHORESIS BY CROSSED VELOCITY FIELDS IN A SUPPORTING MEDIUM

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FIL. LIC., GÖTEB.

AKADEMISK AVHANDLING

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Continuous Zone Electrophoresis by Crossed Velocity Fields in a Supporting Medium

By Inger Brattsten

The present investigation treats of a method of resolving mixtures of dissolved substances of the serum protein type into single components. The separation is conducted on a preparative basis as a continuous procedure by electrophoresis in a supporting medium.

This work was started in 1949 in collaboration with Dr. Harry Svensson, Stockholm. In the course of our collaboration, which extended throughout the years 1947–1949, he introduced me into this field of science, revealing by way of our day by day discussions the ideas and aspects treated by himself and contemporary workers. This bulk of knowledge, which is approximately covered in the references 1–24, also helped to make available to me currents and results of the biochemical research initiated by The Svedberg and proceeding at the Institute of Physical Chemistry, Uppsala, during several decades. Because of their special significance for the present investigation, I want to mention in this connection some fundamental papers by Tiselius (25, 26) and that by Blix, Tiselius, and Svensson (27).

My investigations have been carried out at the Institute of Biochemistry, Uppsala, and to the Head of the Institute, Professor Arne Tiselius, I am most grateful for financial support from grants to the Institute (from the Swedish Natural Science Research Council, the Wallenberg Foundation, and the Rockefeller Foundation) and for laboratory facilities put at my disposal. In particular, the support of the Swedish Natural Science Research Council in 1953 has been essential for the accomplishment of the work. Equally essential have been the skill and interest of the members of the workshop, where important parts of the apparatus have been constructed. The kindness and readiness to help on the part of the staff of the Institute have greatly facilitated my work there.

General Principles

The basic principles employed in the fractionation process are given in the article *An apparatus for continuous electrophoretic separation in flowing liquids* (in collaboration with H. Svensson) (28). The method is conveniently explained with the aid of Fig. 1, taken from that paper.

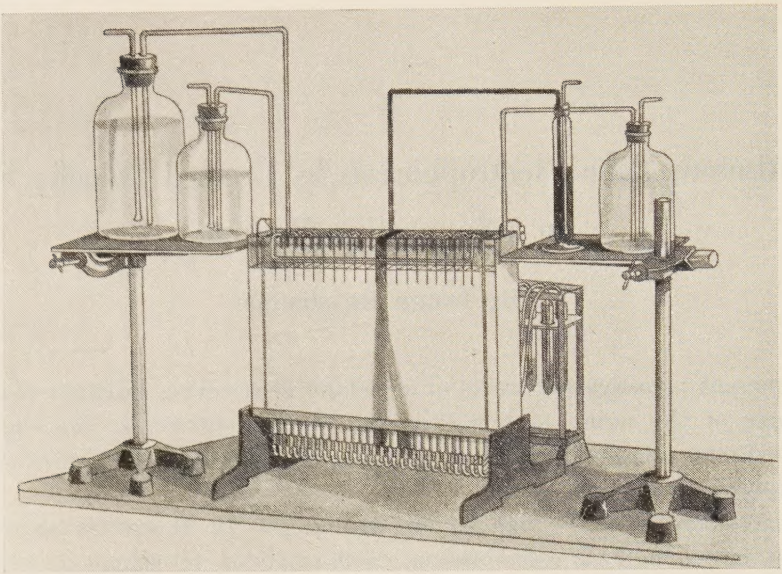

Fig. 1. *The original experimental arrangement.* (From SVENSSON and BRATTSTEN (28).)

In this figure our first apparatus and the original experimental arrangement are sketched (spring 1949). The elongated perspex trough, where the fractionation process takes place, is seen in the centre. The cuvette was open at the top although divided into compartments to allow a regulated access of liquid and equipped with a number of outlet tubes at the bottom. It was filled with a packing of glass powder of approximately uniform size. By the supply of liquid from the Mariotte flasks, seen to the left and right of the cuvette, and simultaneous withdrawal at the bottom exits, a continuous flow of liquid was obtained in the glass powder column. The outflowing portions of the liquid were collected into a series of test tubes, the rate of flow being regulated by adjustments of the pressure head. By addition of suitable amounts of electrolytes to the liquid medium and insertion of platinum electrodes inside the lateral walls, an electric field could be applied in the horizontal direction.

Principles of crosswise flow and continuity

The drawing in Fig. 1 shows the separation of two coloured substances. In addition to the background electrolyte the coloured solution contained a mixture of the two substances to be separated. This mixture was injected into one top compartment only, whence it descended into the flow field as a zone of 5 mm initial breadth. If a regular liquid flow were arranged in the vertical direction but no electric current flowing, the coloured solution would form a vertical band extending from the top to the bottom of the cuvette (cf. Fig. 3, ref. 28). If the liquid flow were stopped after the coloured mixture had reached the bottom plane and the electric flow started in

the horizontal direction, each ion species present would assume a characteristic velocity in this direction. Having different electric mobilities, the coloured substances would constitute two vertical zones of different velocity, gradually moving apart by parallel displacement in the direction of the current. When both flow fields are applied simultaneously, each ion acquires a velocity in the force field, the direction of which depends upon the ratio between the component velocity in the vertical direction, which is constant throughout the column, and that in the horizontal direction, which is characteristic of each ion species. A mixture of substances, injected as a single zone, will accordingly become resolved into a series of component zones, the inclinations of which differ proportionately as the mobility differences of the substances. By constant supply of material the fractionation can be conducted as a continuous process.

Principle of stabilisation

Each component zone in the separation column has a composition different from that of the conducting medium. In general, the density is higher within these zones than in the surrounding solution. Unless the resulting pressure gradients at the zone boundaries are compensated by some means, convection currents will arise, tending to eliminate the concentration differences built up by the current. An ordered separation into pure zones was obtained by supporting the conducting liquid by a capillary system, which eliminated disturbances due to convection.

Regulation of the process

In order to provide a satisfactory support for the flowing liquid, the packing material should be highly homogeneous with regard to the distribution of the free space and electrically inert. The intersticies between the powder particles form a network of capillary channels, where the dissolved substances are transported. The irregular size of these channels necessarily introduces variations of the linear velocities. However, if the average velocity if referred to a volume element of the packing, which is not too small, e.g. 1 cm^3, it may have a sufficiently constant direction and magnitude. From the operational point of view the volume velocity of the liquid in the vertical direction and the total horizontal displacements in the direction of the current are particularly important quantities. They are directly recorded in the experiment and serve to define the working conditions. For convenience, the vertical and horizontal velocity components have been distinguished as the "hydrodynamic" and "electric" velocities, respectively, although any strictly physical definitions have not been applied to these terms.

The experimental conditions with regard to the dimensions of the force field, the size and shape of the capillary spaces, and the conductivity of the ionic medium determine the electric resistance in the path of the current. In a certain system, where these variables have defined values, the total horizontal displacement of a

component zone is directly proportional to the mobility of the migrating substance, the time of transfer, and the electric current. For the efficiency of the fractionation process in continuous operation it is a fundamental requirement that the total displacements remain constant during the time of the experiment. Regulation is possible by adjustments of the current and the time of transfer. When the latter variables have been fixed, the resolving power of the system becomes a defined quantity and the displacements are functions of the mobilities of the migrating substances only. Since the mobility is the discriminating property of the substances to be separated, the resolving power of a reported system is suitably expressed as the mobility difference which causes a maximum separation of 1 cm. If the conductivity of the medium is too low to allow mobility determinations on mixtures by the moving boundary method, a more significant expression of the resolving power can often be obtained when it is related to mobilities valid under standard analytical conditions instead of those prevailing in the fractionation column.

When a certain electric load is applied across the bath electrodes, complete resolution of two substances of different mobility is obtained if the volume velocity in the vertical direction is equal to or less than the difference in volume velocity of the two species in the direction of the current. In practice, allowance must always be made for the broadening of the zones during the migration.

The starting conditions of the present work have been briefly outlined in a subsequent paper, reference 29. The major argument was the need of pure substances, such as proteins. The potentialities of electrophoretic separation were apparent; but also the inconveniences in the practical arrangements used in preparative electrophoresis, in particular the moving boundary technique. Further, the work by CONSDEN, GORDON, and MARTIN in 1946 (22) had brought to general attention the usefulness of a capillary support for stabilising liquids in electrophoresis. Hence, our immediate idea of the apparatus was as a tool in the preparative work, in the laboratory and in larger-scale production.

Almost simultaneously with the work reported in reference 28, an apparatus closely comparable to ours with regard to performance and construction was presented by GRASSMANN and HANNIG (30, 31). Related procedures have later been developed in various other laboratories and applied to serum investigation, e.g. by DOUGLAS[1] and BERRY[2], and to fractionation of hydrolysates of amylose by MOULD and SYNGE (33). The latter workers have achieved a considerable efficiency in the process and could report detailed results based on analysis of the isolated fractions (33, 34).

The principle of continuous separation of substances has also been applied to filter paper electrophoresis. Apparatus based on clamping of paper sheets between glass plates have been constructed by BRATTSTEN and NILSSON (35) and STRAIN and coworkers (36, 37). The

[1] Personal communication by Dr. H. W. Douglas, University of Liverpool, England.

[2] Personal communication by Dr. Hans Hoch, Medical College of Vlrginia, Richmond, Va., USA; cf. ref. 32, p. 399.

technique of carrying out electrophoretic separation in a moist chamber according to WIELAND and FISCHER (38) has been adapted to continuous operation by DURRUM (39) and GRASSMANN and HANNIG (31). Tools of this kind have rendered valuable information in some recent investigations, for example, analysis of snake venoms (40) and purification of plague toxin (41). The application of the paper sheet in a moist chamber involves the advantage that the extraction of heat from the paper is aided by evaporation and convection. However, the evaporation process gives rise to concentration and velocity shifts, which must obviously be controlled during the experiment.

An apparatus similar to that described in reference 28 has recently been introduced by BRADISH and SMART (42). Since they omit the continuous transport by the hydrodynamic flow during the separation process, their method is rather to be related to the batch procedures which have been developed in various laboratories (43, 44, 45, 46).

It is obvious that the possibility of conducting the process in a continuous operation has its greatest practical importance in large-scale preparation work, but also that this application is conditioned by the requirement of an adequate process control. In a small-scale apparatus, the attainment of a high degree of resolution in experiments of limited duration is generally the fundamental constructional problem, and more important than a constant and reliable performance in extended work. Accordingly, simple arrangements often suffice in small-scale procedures, but the technique can be improved by the introduction of special means for maintaining the steady state in the flow field. For example, DURRUM points out the advantage of using a stabilised current even tn the case of paper strip experiments (47); DICASTRO and SAN MARCO stress the importance of eliminating field strength variations caused by a non-constant ion distribution in the medium (48).

Plan of the Work

The preliminary studies reported in reference 28 gave two important results. Firstly, the experimental facts confirmed the validity of the principles applied and strongly indicated that a fractionation method of the expected performance could be developed by further work. Secondly, information was gained as to specific details in the construction of the apparatus and the relative importance of the various factors which influence the constance of the process. Practically all the main problems, which had to be solved, could be distinguished in the preliminary stage of the work, and to a large extent the subsequent development work has been based on direct experiences from the introductory experiments.

The subsequent work has been directed to find a system suitable for separation of substances of the serum protein type. The size of the apparatus has been chosen according to the requirements of convenience and yield under ordinary laboratory conditions. Such solutions of the problems have been pursued, which allow adaptation of the present arrangements at a larger scale process.

In order to achieve operational efficiency, the separating effect of the mobility differences should be enhanced as far as possible by proper choice of working conditions. Simultaneously, the processes of remixing during the transfer should be reasonably depressed. The former effect has generally been attained by decreasing suffi-

ciently the ionic strength of the conducting medium. The processes of remixing fall into two main categories; namely, those essentially depending upon *the properties of the conducting and supporting media*, and those essentially due to *variations in time of the two fundamental velocity processes*. The development of reliable working conditions largely consists in the elimination of disturbances which cause remixing. Favourable static characteristics can only be obtained when all the dynamic equilibria in the system are under control. This control is especially important in the case of the electric transfer. Of the power expended, only a small fraction is consumed in the separation process proper. The main part of the electric current is conducted by the buffer ions and gives rise to various kinds of electrochemical reactions in the system and production of heat. These processes involve the transfer of considerable amounts of energy and must be regulated as well, if constant migration of the protein ions are to be attained.

The regulation of the principle variables in the fractionation process has been treated in the articles *The arrangement of a constant liquid flow and Description of a simple pump apparatus* (29), *The electric control of the fractionation process* (49), and *Fractionation of protein mixtures and Description of the cuvette* (50).

Under the conditions adopted sera and related fluids have been fractionated (50). In order to get an orientation as to the distribution of the serum protein components in the fractions, the latter have been examined by some physico-chemical means. The results of this investigation are reported in the article *Analysis of protein fractions obtained from normal human serum* (51). Two applications of the method on actual medical problems are described in the papers *The distribution of reagins in the serum protein fractions obtained by continuous zone electrophoresis* (in collaboration with H. Colldahl and H. Laurell) (52), and *Analysis of human acute phase protein* (in collaboration with P. Hedlund) (53).

An apparatus for continuous fractionation in a small scale has also been constructed. An account of this work is given in reference 35, *A study in continuous zone electrophoresis in filter paper* (in collaboration with A. Nilsson), cf. reference 49, p. 221.

The Supporting Medium

In reference 28, p. 403, some important properties of the packing material have been briefly specified. When the fractionation is to be effected by controlled electrophoretic migration exclusively, any chemical interaction between the solid and liquid phase is principally a disturbance. Therefore, the effect of charged groups in the solid phase should be eliminated as far as possible.

Our original choice of glass powder as the stabilising agent was based, inter alia, upon determinations of the electro-osmotic flow rate under conditions similar to those prevailing in a fractionation experiment. Aluminium oxide, "Filter cel", sea sand, paper pulp, cotton, barium sulfate, and barium carbonate were tested in ad-

dition. In all the experiments the nature and concentration of the ion constituents of the liquid phase had an apparent effect on the flow rate. When the electronegative character of the pure solid was relatively weak, e.g. in a cotton packing, it was possible to shift the direction of the flow towards the anode by the addition of bivalent cations to the surrounding medium. Ballotini beads were not available at the time of this investigation (1949). The affinity of the serum proteins to powdered glass has been studied by adsorption experiments similar to those reported by SHEPARD and TISELIUS (54). With six various types of glass of known composition significantly different results have been obtained.[1] It is intended to complete the adsorption experiments with a study of the electrokinetic properties and the general chemical resistance of the same material.

Some experiences of glass powder as the supporting medium in fractionation experiments have been reported in reference 29. A more recent experience may be added here.

In a fractionation of cerebrospinal fluid in order to separate the fast trace components from albumin (55), a concentrated solution of the fluid collected *post mortem* in a case of *Hydrocephalus internus infantilis* was used as the starting material. This preparation contained a substance—apparently of lipoid nature—which was strongly adsorbed to the solid phase in the fractionation column and gradually coated the glass particles with a hydrophobic film. As a result of the accompanying velocity disturbances in the upper part of the column, the protein zones were slowly displaced towards the anode during the fractionation. When 405 ml of protein solution had been injected the experiment had to be broken. (A two cm zone was used.) By collecting the fractions in relatively small portions, the backmixing could be restricted to contamination of the faster substances with albumin. After the end of the experiment the cuvette was emptied; hot alcoholic potassium hydroxide had to be used for the removal of the adsorbed material from the glass powder. In a medium of low porosity this separation would obviously have been impossible.

When a high degree of stabilisation is wanted, e.g. when slow rates of transfer are used in long duration experiments, packings of cellulose powder and potato starch have yielded satisfactory results, cf. reference 50. Starch has been more frequently used than cellulose powder because it is easier to pack. In a starch column, coloured substances chemically related to bromphenol blue are strongly adsorbed and cannot be used in the pilot bands. A phycoerythrin zone is usually applied instead, although the adsorption of this substance is not negligible either (in acid media; at high ionic strengths).

Since the electro-osmotic flow is largely cancelled in the present apparatus, specific disturbances of the migration conditions due to electrokinetic effects can hardly be distinguished. There is evidence that a considerable pressure gradient is developed by the current in the low-conducting media, which are commonly used, and flow disturbances occur when the current is closed; during steady state operation the migration paths are strictly linear.

[1] These samples of glass have been prepared at the Glass Institute, Växjö, Sweden, by the courtesy of Dr. BO SIMMINGSKÖLD.

In the course of this work numerous attempts have been made to estimate the degree of adsorption of the serum proteins in the fractionation column. Such determinations are complicated by the special conditions of the experiment. For example, each of the leads to and from the cuvette has a volume of several milliliters and is wide enough to allow mixing of the liquid by convection. A greater trailing of the protein zones has never been observed than could be explained by the mixing in the channels connecting the cuvette and the collecting tubes. Within the limits set by the variation of the rate of transfer, the first traces of protein material in the fractions are always detected at the time expected. When an experiment proceeds under constant conditions, estimations of the concentration in the injected solution and the fractions generally indicate full recovery of the proteins. These statements apply to columns of glass powder, starch, and cellulose powder when the conducting solution consists of an acetate or veronal buffer. It appears that a more refined technique is required for the disclosure of specific adsorption effects, which are certainly present.

The Conducting Medium

Fractionation experiments have been carried out in media of acetic acid solutions and buffer solutions containing the sodium salt and free acid of the following substances: acetic acid, glycine, phosphoric acid, cacodylic acid, diethylbarbituric acid (veronal), and boric acid. In all instances the principles reported in reference 50 have been employed for the arrangement of a separation field of constant composition.

Since the work has been preferentially directed to fractionation of the serum proteins, veronal buffers (15) have been most frequently used. The experiments described in references 50 and 51 give an account of the resolution under the reported conditions. In these experiments the rate of transfer of the liquid was generally slightly enhanced in the immediate vicinity of the end membranes by the use of wide tubes in the pump apparatus (cf. reference 29, p. 518). The protein zones have been dispersed across the separation field until they reached the concentrated end zones. In most instances the conductivity was moderately increased in the first and last few fractions containing protein. As a result of this, the rear γ-globulin boundary usually was abnormally sharp. It is probable that the proximity of the end wall also induced a certain retardation of the advancing albumin zone: the overlapping of the α_1-globulin zone by albumins is apparent in the fractionation diagram of normal serum, cf. Fig. 1 a, reference 51. However, conductivity records indicate that the protein ions themselves introduce changes of the migration conditions, cf. Fig. 1 d, loc. cit. Partly at least the observed overlapping may be due to changes of the protein velocities related to the systematic conductivity variations.

The low conductivity in the media used, about $1 \cdot 10^{-3}$ ohm^{-1} cm^{-1} (at 0° C), obviously favours the resolution of the fast trace components from the slower albumins. In the present apparatus separations of this type are difficult because of the

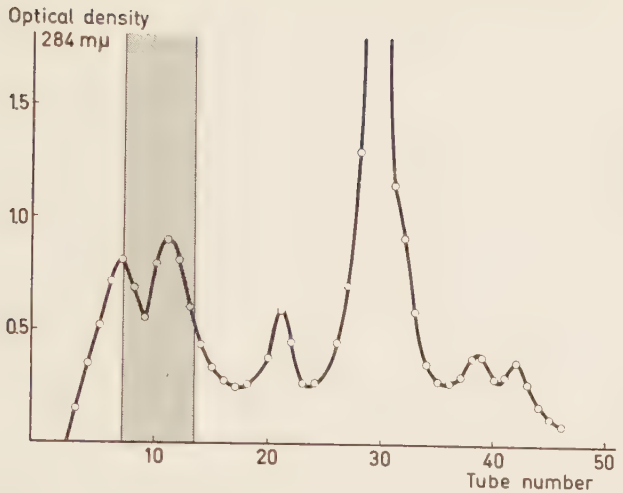

Fig. 2. *Fractionation of normal human serum at pH 8.6.* Conducting medium: veronal buffer of ionic strength 0.03 (tube Nos. 3–7 and 14–49) and 0.05 (tube Nos. 8–13.) The supply of the more concentrated solution is indicated by the shaded zone in the diagram.

limited space in the cuvette in the horizontal direction. Some recent experiments on a normal serum in a field with an applied conductivity gradient gave a distribuiton of the fast substances into two minor peaks in front of the albumin zone, see Fig. 2. In this case an 8 mm broad protein band was conducted through a zone of increased ionic strength as indicated in the figure. A similar result was obtained when the conductivity gradient was due to a controlled penetration of the acetate ion into the separation field. It could be confirmed by paper electrophoresis that the two fast components had different mobility and migrated faster than albumin. The arrangement of such conductivity gradients requires careful adjustments of the experimental conditions, and this method is not equally easy in practice as separation in a field of constant conductivity. Therefore, a certain extension of the field in the horizontal direction is indicated.

Extension of the flow field in the horizontal direction would also involve possibilities of using a higher conductivity in the separation zone. Since the ionic strength of the medium exerts a pronounced influence on the specific interactions between the ions present, it cannot be excluded a priori that the relative mobilities of individual protein components in the fractionation field are different from those prevailing under analytical conditions. Specific changes of the migration conditions caused by the low ionic strength in the medium used—about 0.03—appear to provide a possible explanation of observed deviations from the normal behaviour. Many indications have been obtained that individual electric mobilities can be changed in a desired direction by an appropriate choice of the ionic strength of the medium.

The Velocity Control

As stated already, the regulation of the velocity processes serves to eliminate such variations in time which would alter the net displacements of the substances to be separated, thus reducing the efficiency of the fractionation.

The regulation of the hydrodynamic velocity is treated in reference 29. In order to eliminate flow disturbances due to external pressure variations, the fractionation column is wholly enclosed in the trough and connected with a free liquid surface at one point only. The construction of the cuvette is described in reference 50. The electrode vessels, which are open at the top and percolated by an electrolyte solution, are separated from the middle compartment by highly water-impermeable membranes. Provided the mechanical resistance in the packing is uniform, a regular flow is obtained when liquid is pumped into the cuvette by the top openings, which are 10 mm apart, and withdrawn at the bottom exits at 5 mm intervals. The pump apparatus is described in reference 29. The rate of the liquid flow is regulated by adjustments of the field of the d.c. motor which drives the pump apparatus.

The regulation of the electric velocity has been treated under three main headings. The rate of transfer of the protein ions depends upon their mobility and the prevailing field strength, and all processes must be regulated, which influence these variables. The transfer of the ions in the conducting medium (i) is treated in reference 50; the transfer of heat (ii) and the direct current control (iii) in reference 49.

(i) The transport of electricity through the membranes separating the fractionation and electrode compartments is effected by sodium and acetate ions. It has been found that these ions pass sufficiently tight membranes of cellophane or parchment paper without causing any perceptible disturbances of the fractionation process. The exchange is facilitated by a high salt concentration in the solutions in immediate contact with the membranes.

In the fractionation field high rates of migration have mainly been accomplished by the use of relatively dilute media Such systems are very sensitive to contamination with foreign ions, especially those of high mobility. The appearance of a conductivity gradient in the separation field is accompanied by equilibrium shifts in the operating processes. A twofold effect can be distinguished: firstly, the migration velocities of the protein ions are changed in direct proportion to the field strength and mobility changes; secondly, by Ohm's law the resulting change of the electric resistance in the cell introduces a new voltage-to-current level and hence a shift in the power balance in the system. The application of the "reservoir zones" of concentrated solutions at both sides of the separation field has proven an effective means of impeding the migration of conductivity gradients directed towards the centre part of the cell. However, space conditions require that the reservoir zones are dimensioned relative to a maximum rate of transfer of electricity, which must not be exceeded.

(ii) The transfer of heat in the system is regulated by means of special arrangements for the cooling of the cuvette and by control of the electric power. Since relatively great quantities of electric energy are involved in the procedure adopted, the second method is indispensable. In order to control the power, both the current and the voltage should be regulated. In practice, an adequate control of the process is obtained by continuous regulation of the bath current and supplementary control of the voltage across the bath electrodes by means of a relay, which breaks the current, should the voltage increase abnormally. Such an increase of the voltage would result if the resistance in the cell accidentally increased, e.g. by shortness of supply liquid. An event of that kind need hardly occur; under ordinary conditions the continuous regulation of the bath current provides a sufficient stabilisation of the process. A reversed arrangement, i.e. a process control on the basis of a constant supply voltage, is generally invalidated by the inertia in the extraction of heat from the cell and difficulties to avoid peaks in the current which cause penetration of salt gradients into the zone of high resistance (cf. the preceding section). A resulting decrease of the cell resistance in this case tends to increase the power expended and hence the energy level in the cell. Experience has shown that autoinduced shifts in the electrochemical equilibria may rapidly degenerate into a dangerous cycle unless the power is properly controlled.

(iii) In order to cancel fluctuations of the net displacements due to minor variations in the rate of transfer of the liquid, the bath current is regulated to follow the shifts in the pump speed. The regulation is actuated in a two-stage transductor regulator, described in reference 49, the Appendix (by L. Lannefors, ASEA, Västerås, Sweden). This tool has been used in the fractionations described in references 50–53.

Applications

The introduction of current control by means of the transductor regulator (Jan. 1953) essentially improved the constancy in the process and simplified the attendance on the experiments. Hence it became possible to apply the method to some actual problems of blood investigation. The present studies, which are analytical and largely tentative, have been performed on normal and pathological serum and cerebrospinal fluid.

By fractionation of a fluid of biological origin and subsequent analyses of the collected fractions a component of specified activity—either normal or pathological—can be detected and characterized with regard to its general chemical and physicochemical properties. This procedure requires in the first place the application of sufficiently great quantities of material and a satisfactory resolution of the components present in the mixture. Further, since biological substances are generally very sensitive to denaturing agents, drastic changes in the equilibrium conditions must be avoided, and the different steps in the investigation should be carried out within a relatively short time, while the material is fresh.

In particular, the isolation of trace components from a complex mixture involves considerable demands on the capacity of the method. The experiments reported in reference 50 show that trace components of higher mobility than albumin can be separated from this substance in serum and cerebrospinal fluid at pH 8.6 and serum at pH 4.5. Four different sera have been investigated at pH 4.5 5.0, and in all instances one or two small peaks were present on the anode side of the major peak in the fractionation diagram. It is probable that these components are related to the substances recently analyzed by Winzler and coworkers (56, 57, 58), although this has not been confirmed by experiments. The isolation of trace components on a preparative basis has instead been studied in fractionations of cerebrospinal fluid at pH 8.6. Portions of 1500 ml of normal fluid (pooled material) were found to give 5–7 mg of the front component. One preparation of this kind could be accomplished to within five days after the fluid had been obtained from the hospital. Since both the starting material and the isolated protein fractions were originally present in very dilute solution (about 0.04 and 0.01 percent of protein, respectively), the success in the work largely rested upon the method of concentrating the material by removal of water and dialyzable salt ions. This part of the investigation and the examination of the isolated material is due to Aly. In reference 55 he describes the concentration procedures and some preliminary fractionation results. The fluid was generally concentrated by dialysis against a preparation of high polymer dextran in concentrated solution, the isolated protein fractions by ultrafiltration. Later analyses have shown that dextran particles generally penetrate into the dialysis bag during a concentration dialysis. The contaminating substance could not be removed by prolonged dialysis against dilute buffer solutions. However, the migration of the protein ions in the electric field apparently takes place independently of the dextran particles present in the injected mixture. In subsequent experiments (see below) the concentration of the serum fractions for the analyses was carried out either by ultrafiltration at 4°C or pervaporation by a rapid stream of air in contact with narrow cellophane tubes which contain the dilute protein solutions. The first method is considered the gentler one, although it is more time-consuming than the second (rate of filtration about 4–6 ml per hour).

As a result of the preliminary fractionation experiments on cerebrospinal fluid, evidence has been obtained that the material isolated in front of the albumin zone contained at least two components of slightly different mobility and sedimentation constant, cf. reference 55. This is in agreement with the findings of Hoch and Chanutin (59). They have further established the existence of several trace components in front of the rising albumin boundary when serum is analyzed by the moving boundary method under special conditions (60). The recent finding of two front components in the fractionation diagram of a normal serum is also referred to, see Fig. 2 in this paper.[1] Additional studies are needed to reveal the identity of the

[1] This serum was obtained from a donor in the blood bank of the University Hospital, Uppsala.

fast trace components present in serum and cerebrospinal fluid, in the normal and diseased state. The results hitherto gained indicate that satisfactory conditions can be derived for their isolation by continuous zone electrophoresis.

In comparison with a preparation of the type described, the isolation of singular serum components of intermediate mobility involves greater difficulties. The analyses of the serum fractions reported in reference 51 reveal the complexity of the pattern, especially in the mobility range of the β- and α-globulin constituents. It is evident that direct chemical analyses of the reported fractions, e.g. with regard to their carbohydrate or lipid content, would contribute little to the characterisation of the individual proteins present. Therefore, such analyses are planned to be carried out on preparations obtained under different conditions, which give a higher resolution.

Since the physico-chemical analyses give information about the general distribution of the serum components under the reported conditions, they provide a useful basis for any further differentiation of the method needed to meet the practical demands in various cases. Further, the information gained by the analyses has a direct importance in those cases where the reported conditions can be applied without modifications, e.g. when the resolving power of the system allows complete separation of the substances investigated or when a problem can be solved without studies on pure substances. For example, if a specific pathological component is present in a fluid under investigation, the behaviour of this component in the fractionation field can be related to that of the normal constituents. When the distribution and properties of the latter are known, the comparative information gained allows a certain characterization of the foreign substance.

Two analyses of pathological sera by the method described are reported in references 52 and 53. They refer to conditions of allergic asthma and pneumonia in the acute stage, respectively. In both instances the fractionation diagram showed a pronounced increase of the α-globulin component, but the reactive substance was recovered among the γ-globulins. In the first case the activity could be traced into the range of the β-globulins (see Table 1 in reference 52); in the second case the titration values closely followed the γ-globulin curve (see Fig. 1 in reference 53). The distribution of the reactive substance relative to the normal serum constituents in both cases gave evidence of the heterogeneity of the material. The reagins present in the blood in cases of an allergic disease are known to react with specific antigens, but the chemical nature of the reacting substances is unknown as yet. The action of the acute phase protein is reported to be unspecific; both the nature of this substance and its clinical function are largely unexplored. The present findings of pathological components among the γ-globulins and a simultaneous increase of certain other globulin components are by no means isolated; for example, Seibert and coworkers have reported a similar development in conditions of tuberculosis (61). The regular increase in the α-globulin content of serum in certain diseases has been treated by Longsworth and coworkers (14). In many instances the pathological

increase of the α-globulin component is accompanied with the appearance of acute phase protein in the blood. This coincidence and the fact that the substance precipitates with the albumin portion in salt fractionation of acute phase serum have contributed to the opinion that the reactive substance is an α-globulin (62). However, the present finding of acute phase protein among the γ-globulins is in substantial agreement with the results obtained by Wood, McCarty and Slater (64).

In view of the previty of the account of this investigation (reference 53), a few details may be added here. In comparison with the protein distribution in normal serum, the acute phase serum showed deviations in several respects. The form and site of the two intermediate globulin peaks in the fractionation diagram are unfamiliar. The distribution of the protein in this record was closely reproduced by filter paper and moving boundary electrophoresis. No splitting of the α-globulin boundary was observed in free electrophoresis. The α-globulin fractions, which in the normal sera investigated have been colourless, in this case possessed the reddish-brown colour characteristic of the normal β-globulin component. Of the two components adjacent to the γ-globulin the faster one was colourless, the slower showed a distinct bluish-green tint. These findings may indicate changes in the composition and specific interactions between the protein constituents, which were caused by the diseased conditions.

By electrophoretic fractionation of acute phase serum, material has been collected for a study of the behaviour of the isolated protein fractions in salt precipitation experiments.

Inasmuch as the aim has been the collection of material for analysis of various kind and meaning, all the fractionation experiments reported in this thesis have had an analytical rather than really preparative purpose. Therefore, any appreciable amounts of material have not been applied in each experiment. The serum fractionations have generally been carried on until 2–5 ml of 1 % solution of the fractions to be analyzed could be prepared. With a rate of fractionation of 1 ml of whole serum per hour and a resolution of the serum components into 45–48 fractions of increasing mean mobility (cf. references 50 and 51) one fractionation experiment lasted 20–60 hours, depending upon the quantity of serum applied.

Although representative of the results obtained, these data by no means give a complete account of the capacity of the present method. In many practical cases the critical mobility differences are greater than in the serum system, thus allowing enhancement of the fractionation rate without any loss in the purity of the isolated substances. This can be achieved either by an increase of the zone breadth of the injected mixture or increase of the rate of transfer of the streaming liquid. Without decreasing the fractionation rate, a higher resolution can be obtained within a mobility range smaller than that occupied by whole serum by simply increasing the electric velocities of the substances to be separated. Simultaneous operation of several cells in parallel would obviously serve to increase the yield per unit time in the process.

Typical of the performance is the simplicity gained by eliminating hydrodynamic velocity, temperature, and conductivity gradients as variables which

may effect fluctuations of the net displacements during the operation. Under the present conditions the process is adequately controlled by the combined, continuous regulation of the bath current and the pump speed. The validity of this control has been tested in long duration experiments. At the present the maximum fractionation rate and resolution are set by the cell size rather than the temperature, and the capacity can undoubtedly be increased by simply increasing the dimensions of the cuvette. All evidence gained in the experimental work predicts that the principles of regulation now applied will retain their validity in continuous operation in a truly preparative procedure.

Summary

On the basis of the fractionation experiments described by Svensson and Brattsten (1949) a procedure for preparative electrophoretic separation has been developed.

This procedure employes a continuous fractionation in a combined electric and hydrodynamic flow field stabilised by a capillary system. The work has been essentially directed to provide conditions suitable for separations of substances of the serum protein type.

A flow field of constant intensity is arranged by mutual regulation of the rate of transfer of the streaming liquids and the bath current. The liquid flow is effected by pumping. In order to cancel variations in the pumping velocity a feedback control of the bath current has been arranged, which maintains a constant ratio between the two variables. The validity of the process control has been ascertained in experiments extending during 10 days and more in continuous operation.

The fractionations are carried out in media of dilute buffer solutions (conductivity 1.0–1.5. 10^{-3} ohm^{-1} cm^{-1} at 0° C.). In a packing of the dimensions $28 \times 30 \times 1$ cm^3 a serum solution, injected at a rate of 1 ml of whole serum per hour, can be divided into maximum 48 fractions of increasing mean mobility. This corresponds to a total separation of the albumin and γ-globulin zones of approximately 20 cm.

The procedure has been mainly applied in investigations of normal and pathological serum and cerebrospinal fluid.

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